Matrix metalloproteinase-9 activity increased by two different types of epileptic seizures that do not induce neuronal death - A possible role in homeostatic synaptic plasticity

Summary of Ph.D. Thesis

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INTRODUCTION

Matrix metalloproteinases (MMPs) are parts of the family of inducible brain proteases. MMP-2 and MMP-9, also known as gelatinase-A and -B, are Zn2+-dependent members of this family with recognized roles in proteolysis of the extracellular matrix (ECM). They degrade ECM proteins, including adhesion and signaling molecules, as well as neurotransmitter receptors and growth factors. Thanks to their synaptic localization, their function is frequently studied in synaptic plasticity. MMP-9 is activated during Long-Teré Potentiation (LTP) and retinal light adaptation. Expression of MMPs increases in pathological processes including tumorgenesis, inflammation and chronic epilepsies. The effects of MMP activation include alterations of neuronal excitability and synaptic efficacy via changes in synaptic structure. MMP-9 is induced during the status epilepticus after kainate or pilocarpine treatment. In these models, MMP-9 appears to participate in the processes of epileptogenesis and contribute to neuronal death, pruning of dendritic spines and forms of aberrant synaptic plasticity leading to chronic recurrent seizures. However, in human epilepsies, only a small percent of patients develop chronic epilepsy, probably due to the processes of homeostatic synaptic plasticity which initiates plastic changes that tend to counteract any alteration in neuronal excitability. MMP-9 may participate in such a protective homeostatic plasticity via its extracellular substrates, which include the integrins, cadherins or β-dystroglycan. MMP-9 also influences synaptic signaling by acting directly on both NMDA and AMPA subtypes of glutamate receptor. These changes would act homeostatically to reduce the efficacy of glutamatergic synapses after seizures. The lower efficacy of kainate in producing secondary generalized seizures in genetically epileptic rats and the reduction in ictogenic potential of 4-AP observed in kainate-treated chronic epileptic rats may also suggests that homeostatic plasticity can be initiated after seizures.

However, it remains unclear whether the enzymatic activity of MMP-2 and MMP-9 is enhanced by epileptiform activities that do not induce neuronal death and consequent recurrent spontaneous seizures. To answer this question, we therefore used two different animal models of epilepsy that do not involve cell damage and aberrant synaptic plasticity. In the first model we induced generalized cortical seizures by focal application of the K+ channel blocker 4-aminopyridine (4-AP). The second genetic epilepsy model was the Wistar Glaxo Rijswijk (WAG/Rij) rat. Both model could be valuable in studies on homeostatic functions of MMP-9 activity in the epileptic brain.

THE AIMS OF THE STUDY

1. We studied gelatinase activity patterns of different brain areas in the acute 4-AP model to find out weather gelatinases are activated in epileptic activity that doesn't lead to widespread neuronal loss, aberrant synaptic plasticity and thus spontaneous recurrent seizures.

2. We measured basic gelatinase activity during development in the genetically epileptic WAG/Rij and in the healty SPRD rat strain to get more information about the differences between age groups. We also studied the differences in the gelatinase activity between the genetically epileptic WAG/Rij and in the healty SPRD rat strain to answer the question whether the genetically epileptic brain shows a different gelatinase pattern than normal brains.

3. We studied the daily gelatinase activity changes in WAG/Rij rats to see if it can be correlated with the daily pattern of SWD genesis.

MATERIALS AND METHODS

Animals and reagents

Experiments were carried out on male WAG/Rij (Wistar Albino Glaxo/Rijswijk, Netherlands) and Sprague–Dawley (Charles River Laboratories, Hungary) rats. Animals were housed in groups of 3–5 in standard conditions of a 12 h light/dark cycle (light from 06:00 h till 18:00 h). Animal care and treatment conformed to guidelines of the Council Directive 86/609/EEC and the Hungarian Act of Animal Care and Experimentation (1998, XXVIII), and with local regulations for research animals. We took care to minimize pain, suffering and the number of animals used.

Surgery procedures for 4-AP administration

4-AP experiments were carried out on male Sprague–Dawley rats (300–400 g) which were anesthetised by halothane and placed in a stereotaxic frame. A hole, of diameter 1.5–2 mm, was drilled in the skull above the parietal cortex (coordinates from bregma: anterior: 5.8, lateral: 3.2). After 30 min recovery, 4-AP (0.5 mg/kg) was placed on the parietal cortex surface. After 45 min,

the cortical surface was washed out, the wound was closed and animals removed to a quiet, dark and secure place for observation of the behavioural symptoms of epilepsy.

Tissue dissection

Animals were decapitated under halothane anesthesia (1–1.5% halothane in air), and brains dissected in cooled ACSF (artificial cerebrospinal fluid). Ipsi- and contralateral parietal and frontal cortices, hippocampi and thalami were dissected on an ice-cold plate. Tissue was stored at 80 °C before analysis. Animals were decapitated at 12:00 h, during the low-frequency seizure period for WAG/Rij animals, except 4 rats sacrificed at 18:00 h during the high-frequency seizure period. Ipsilateral and contralateral parietal and frontal cortices, hippocampic and thalamic tissue samples were prepared on ice : from 4-AP treated and sham operated control rats at three time points: t = 0 corresponding to the end of the 4-AP application, t = 6 h and t = 24 h (n = 6 for each group) ; from 4 WAG/Rij and 4 Sprague–Dawley rats aged 5–6 weeks, 4 WAG/Rij and 4 Sprague–Dawley rats aged 5–7 months; from WAG/Rij rats sacrificed at 12:00 and at 18:00.

EEG recordings.

Screw electrodes were inserted above the frontal, somatosensory and parieto-occipital cortices through holes drilled in the skull with a wound clip placed under the skin as reference electrode. A hole was drilled to expose the parietal cortex for 4-AP application and a Teflon rod was inserted so the hole remained accessible. After 1 week, a baseline EEG was recorded, and under halothane anesthesia the 4-AP was applied. EEG records were made during 6 h after 4-AP application and for 1 h periods at 12 h and 24 h and 7 days. Animal behaviour after 4-AP application was scored according to the Racine scale with modifications.

In 3 WAG/Rij animals prolonged (11:00 h until 19:00 h, 8 h) EEG records were made to follow the circadian variation in the occurrence of spike-wave-discharges (SWDs). Since animals were maintained on a 12 h) light/dark cycle, this time included a period of sustained illumination followed by a transition to darkness. Procedures were similar to those for EEG records from 4-AP treated animals with screw electrodes inserted above the parietal, fronto-parietal and frontal cortices and identical EEG settings. Changes in SWD frequency were estimated with respect to the frequency between 11:00 h and 12:00 h. The absence of seizures were also confirmed with EEG records from 2 WAG/Rij animals aged 6 weeks.

Gelatin zymography

Samples of the different brain areas were homogenized in ice-cold buffer, insoluble supernatant fractions were precipitated with cold 60% ethanol and centrifuged. Precipitates were solubilized in nonreducing SDS-sample buffer and incubated for 15 min at 37 °C. Protein concentrations were measured and equal amounts of proteins from different brain areas were separated on 7.5% SDS-PAGE gel copolymerized with 0.1% FITC-labeled gelatin. Enzymatic activity causes dark bands with a bright fluorescent background on the gels (in contrast to Coomassie stained gels). Proteinase activity was quantified by densitometry of gelatinolytic bands. Gelatinase levels from treated rats were compared to control samples loaded on the same gel. Density differences were analyzed by a one-way ANOVA test with post hoc multiple comparison test (Tukey test). The gelatinolitic bands were validated also by western blot analysis

***In Situ* zymography**

The control and 4-AP treated brains were frozen immediately in dry ice after decapitation and stored at -80°C until cryosections of 10-µm were made at -22 ºC in the sagital plane. The frozen sections were incubated for 72 hours at 37°C in a humid dark chamber in 100ml of reaction buffer containing 100µg/ml of FITC-labelled DQ-gelatin (EnzCheck Gelatinase/Collagenase Assay Kit). As control we incubated the sections in only the reaction buffer without gelatin substrate. After washing in PBS, fixing in cold PFA (4% in PBS) and mounting in Mowiol we detected gelatinase activity with fluorescence microscopy (Ex: 495 nm, Em: 515 nm).

RESULTS

1. The 4-AP model

1.1. Generalizing cortical epilepsy induced by 4-AP application

Seizures were initiated focally by 4-AP application and then generalized into a secondary status epilepticus involving all cortical regions. At the focus the first paroxysmal events appeared at 15 min and they evolved into continuous spiking activity after 60 min. At this time, similar activity was detected at contralateral sites in parietal cortex, and seizures were generalized to all cortical regions at 3 h after 4-AP application. At 6 h, spectral densities of EEG signals showed a peak in power close to 1 Hz and large (300–350 mV) low-frequency (1–1.5 Hz) events predominated. On emergence from the anesthetic, all 4-AP treated animals exhibited severe epileptic seizures and that reached Stage 5 of the Racine scale. Recovery from the status epilepticus occurred at

24–48 h after 4-AP treatment. At one week, no epileptiform activity was evident in EEG records and rat behaviour appeared to be normal.

1.2. The enzymatic activity of MMP-9 in the 4-AP model

We showed that enzymatic activity of both precursor and active forms of MMP-9 was enhanced in the contralateral parietal cortex, hippocampus, frontal cortex and thalamus, regions of secondary generalization of cortical seizures induced by focal 4-AP application. Changes in active and precursor forms of MMP-9 occurred most rapidly in the parietal cortex and hippocampus immediately after 4-AP application ceased (t = 0 h), both ipsi- and contralaterally. It seems probable that this ipsi- and contralateral elevation resulted from activating proteolysis of existing pro-MMP-9 pools rather than protein synthesis. At 6 h, pro-MMP-9 increased in ipsi- and contralateral parietal cortex and hippocampus by 150-200 %, while there was a significant decrease in active-MMP-9 in the ipsilateral cortex. At 6 h, there were no significant changes in active or pro-MMP-9 in tissue from any other site. We note that the delayed involvement of epileptiform activity as well as potentially distinct mechanisms of MMP-9 regulation may contribute to the delayed upregulation of the zymogen in the thalamus and frontal cortex. Enzymatic levels of both precursor and active-MMP-9 increased further at 24 h. In the ipsilateral parietal cortex, near the site of 4-AP application, pro-MMP-9 increased to 2240 % of control values. Significant, but smaller, increases occurred at sites recruited during seizure generalization. Enzymatic levels of active-MMP-9 increased comparably in parietal cortex, frontal cortex and thalamus and a remarkable (600%) increase was found in the hippocampus, both sides. We noted interesting reciprocal changes in the pro- and active forms of MMP-9 in parietal cortex at 6 h after 4-AP application followed by considerable increases in both forms at 24 h. The reduction followed by rebound in the active form may depend on multiple factors that control the conversion of pro- to active-MMP- 9 or the level of active-MMP-9. These data thus demonstrate significant, delayed increases in both active and precursor forms of the proteinase MMP-9, but not MMP-2, at sites of generalized seizure activity induced by focal 4-AP application. Longer term elevations of the pro-form would tend to produce increased levels of functional MMP-9.

2. WAG/Rij model.

2.1. Enzymatic activity of MMP-9 and MMP-2 in young and adult WAG/Rij and Sprague Dawley rats

Since both MMP-2 and MMP-9 contribute to developmental processes, their enzymatic activity could be expected to be reduced in adult animals. While the enzymatic activity of MMP-2 was largely reduced in the adult, we detected an increase in the pro-MMP-9 pool in the adult for both SPRD and WAG/Rij rats. Possibly the adult brain uses less of the inducible pro-MMP-9 pool than the young brain. Baseline levels of pro-MMP-9 increased considerably in hippocampus, both cortical regions examined and the thalamus between 6 weeks and 6 months, in both SPRD and WAG/Rij rats. This increase was largest in the hippocampus and we assume that it results from developmental mechanisms. However comparison of adult levels of MMP-9 in WAG/Rij and Sprague–Dawley animals revealed much larger differences in the thalamus and frontal cortex which are involved in SWD generation than in the hippocampus

Overall, these data reveal an age-dependent reduction in MMP-2 activity in both WAG/Rij and SPRD rats. In contrast pro-MMP-9 increased significantly between young WAG/Rij animals which did not and older animals which did generate absence-like seizures. Pro-MMP-9 was also elevated in adult WAG/Rij rats as compared to age-matched SPRD animals, but levels of active-MMP-9 were not generally higher in WAG/Rij animals

2.2. Pro-MMP-9 enzymatic activity is enhanced in seizure-generating adult WAG/Rij rats.

We detected some differences in the enzymatic activity of MMP-2 in adult WAG/Rij rats and control SPRD animals. MMP-2 levels were higher in the thalamus and frontal cortex but essentially similar in the parietal cortex and hippocampus. In contrast, levels of pro-MMP-9 activity were consistently higher in adult WAG/Rij rats than in adult control SPRD rats, in every studied area, especially in the thalamus. An enhanced pool of pro-MMP-9 in WAG/Rij rats could provide a rapidly available pool of MMP-9 in one of the generator areas for thalamo-cortical seizures.

Thus, we next searched for dynamic changes in enzymatic MMP-9 activity correlated with the circadian variation in SWD frequency. Spike and wave discharges in WAG/Rij animals consisted of a spindle-like series of EEG waves at 8–10 Hz that were at least 2 times larger than control delta waves. We observed variability in frequency of the occurrence of SWDs during prolonged EEG recordings made from 11:00 until 19:00 h that included a transition from light to darkness. In our animals, the lowest frequency of SWDs (6.32/h) occurred between 11:00 h and 12:00 h and the highest frequency of SWDs occurred between 17:00 h and 18:00 h (17.46/h). Tissue samples to compare MMP activity were prepared at time points corresponding to these periods of high and of low frequency of SWD occurrence. Analysis of the enzymatic activity of these tissues revealed an increase in active form of MMP-9 during periods of high SWD occurence in areas associated with SWD generation: in the thalamus and frontal cortex, but no

change in the parietal cortex. In contrast, pro-MMP-9 activity was decreased in the thalamus and parietal cortex but unchanged in frontal cortex. These results therefore suggest that changes in enzymatic actions of the active form of MMP-9 may vary with seizure frequency in areas involved in seizure generation.

CONCLUSION

Data presented here suggest in two different animal models of epilepsy that synchronized cortical seizures alone suffice to enhance both the precursor form and the active form of MMP-9. Different spatial patterns of changes in pro- and active forms of MMP-9 were detected in response to the thalamo-cortical seizures of WAG/Rij rats and the generalized cortical seizures induced by focal 4-AP application. We detected no comparable increase in enzymatic activity of MMP-2. The first epilepsy model consisted of generalizing cortical seizures induced by focal application of the convulsant 4-AP. We detected both activation and increased synthesis of MMP-9 during and after seizures. It seems likely that these processes are independently controlled and our data do not discriminate between the relative roles of epileptiform spiking or of seizure-induced plasticity. Our previous data revealed no detectable neuronal loss after 4-AP treatment in the studied regions, only sporadic ‘‘dark’’neurons at distant sites up to 6 h after 4-AP application using the Gallyas silver stain which marks cells that seem to recover and do not die. Our data suggests that MMP-9 enzymatic activity is enhanced during this period of neuronal recovery. If so, MMP-9 activation at sites distant from 4- AP application may be associated with the abnormal neuronal discharges corresponding to the epileptiform activity, rather than processes associated with cell death and aberrant synaptic plasticity. Even so, MMP-9 enzymatic activity was maximal near the application site in parietal cortex, where some neuronal necrosis does occur so we cannot completely exclude a contribution from cell death. This hypothesis is also supported by our findings that while in the genetically epileptic rats where no cell death is associated with the emergence of thalamocortical seizures we showed increases in MMP-9 activity in rats aged about 4 months.

The second model was the genetic absence seizure animal WAG/Rij. In these animals MMP-9 activity was considerably enhanced during the age-range at which thalamo-cortical absence-like seizures were first generated. Furthermore, MMP-9 activity showed a diurnal increase in areas involved in seizure generation that was correlated with a time period at the transition from sleep to wakefulness when seizure activity is elevated. Increased MMP-9 activation from its pro-form during periods of higher seizure frequency might result directly from neuronal depolarization, although we note that significant increases in MMP-9 after 4-AP

induced seizure activity occurred with a rather longer latency. Previous work suggests that the constitutive pool of pro-MMP-9 may be rapidly transformed into the active form of the proteinase. Further the pool of pro-MMP-9 seems likely to be dynamically replenished by gene transcription induced by a seizure. We note that the pro-MMP-9 level remained increased at 24 h after seizures induced by 4-AP in SPRD rats and was consistently elevated in WAG/Rij rats at ages after the onset of genetic absence seizures. Conceivably an increased availability of MMP-9 may be functionally important after a seizure. The activation of MMP-9 varied between cortical regions in a way potentially consistent with a differential participation in absence seizure activity. While SWDs are recorded from widespread cortical and thalamic regions, they appear to emerge from facial somatosensory cortex and in general, frontal regions show more, sometimes larger SWDs, than do parietal regions. A correlation between elevated pro-MMP-9 levels in WAG/Rij rats and regions of SWD generation could explain our data showing different levels of enzymatic activation in frontal and parietal cortex

Our data from these two epilepsy models suggests that MMP-9 elevation results from elevated neuronal activity since neuronal death does not occur in the WAG/Rij model of absence seizures and since we detected no neuronal death in zones of propagation of seizures induced by 4-AP. Sustained depolarization induces both pathological and protective changes. The extracellular proteolysis mediated by MMPs may contribute to both types of process. Consistently elevated pro-MMP-9 levels should enhance proteolysis of extracellular matrix components and other extracellular proteins. Proteolytic control of peri-neuronal structures could provide a rapid external pathway to achieve homeostatic changes in synaptic efficacy. Similarly MMP-9 induction following neuronal depolarization may provide a feedback control of cellular excitability. Further studies are needed to specify the functions of elevated MMP-9 enzymatic activity in these two models, but they should be valuable in studies on homeostatic functions of MMP-9 activity in the epileptic brain.

PUBLICATIONS RELATED TO THE PHD THESIS

Takacs E, Nyilas R, Szepesi Z, Baracskay P, Karlsen B, Røsvold T, Bjørkum A, Czurko A, Kovacs Z, Kekesi AK, Juhasz G (2010). Matrix metalloproteinase-9 activity increased by two different types of epileptic seizures that do not induce neuronal death: A possible role in homeostatic synaptic plasticity, *Neurochemistry International* 56, 799–809

Papp AM, Nyilas R, Szepesi Z, Lorincz ML, **Takacs E**, Abraham I, Szilagyi N, Toth J, Medveczky P, Szilagyi L & Juhasz G. (2007). Visible light induces matrix metalloproteinase-9 expression in rat eye. *J Neurochem* 103, 2224-2233.